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PATENT- OG VAREMÆRKESTYRELSEN

Dendrimer conjugates for selective and highly efficient solubilisation of protein aggregates

FIELD OF THE INVENTION

The present invention relates to dendrimer conjugates formed between a dendrimer and a protein solubilizing substance. The dendrimer conjugates are effective in the treatment of protein aggregate related diseases like e.g. prion- related diseases, Alzheimer's etc. The dendrimer conjugates make the aggregates more soluble in a reaction medium, thereby increasing the protease sensitivity of the protein aggregates. The increase in the protease sensitivity is due to a synergistic effect of the dendrimer conjugate, i.e. a physical mixture of the individual components (dendrimer and protein solubilizing substance) will not increase the protease sensitivity to the same extent.

BACKGROUND

Protein aggregates are involved in a number of pathological processes, including prion-related diseases and amyloid-related diseases (Alzheimer's disease, Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, Parkinson's disease, diabetes type II, Huntington's disease and others). These diseases are not very well understood, all are substantially incurable, and all are devastating or fatal. In addition, the prion group of these diseases are highly transmissible and, in one case *viz.* bovine spongiform encephalopathy, even zoonotic, meaning that it can spread from animals to humans. All the diseases belonging to this group are dependent on the formation of protein aggregates formed upon proteolytic cleavage or upon abnormal folding of normal, naturally-occuring precursor proteins. The transmission of prion diseases is thought to occur through unconventional mechanisms, involving the induction by abnormally folded prion protein of abnormal and pathogenic folding in normally folded host prion protein that is normally expressed in the host. In addition, some prion diseases are heritable and are thought to occur through mutations in the prion gene, such mutations facilitating the formation of the pathogenic structural isoform of the prion protein.

One hallmark of the protein aggregates of these diseases is their substantial insolubility in aqueous buffers and their very high protease resistance. In recent years a number of compounds have been shown to be able to dissolve or increase the protease

susceptibility of prion protein aggregates ("plaques") to some extent. These compounds include antibodies, a number of small-molecule drugs (quinacrine and chlorpromazine, amphotericin B, pentosansulfate and Congo Red) and polyamine dendrimers (US 6,214,366 B1, US 2002/0041859, Supattapone et al., 1999, Proc. Natl. Acad. Sci., USA 96, 14529-14534, Supattapone et al., 2001, J. Virol. 75, 3453-3461) and, in some cases, their effect on the transmissibility or "infectivity" of treated prion proteins has also been demonstrated. It is thus known that dendrimers have some effect on their own: certain dendrimers (especially cationic dendrimers) can interact with prion aggregates and partly dissolve them, to a state where they have become protease sensible and lose infectivity (Suppatapone et al. 2001, see above). Interestingly, such dendrimers were shown to be able to both inhibit aggregate formation and to dissolve already formed aggregates to an extent where, for example, a cell culture forming prion protein aggregates were "cured" of these aggregates. Older literature suggests that scrapie is sensitive to thiocyanate, trichloroacetate and hydroxyl ions (Prusiner et al., 1981, Proc. Natl Acad. Sci., USA 78, 4906-4910).

It is likely that these compounds exert their effects through different mechanisms including effects on the prion protein expression in live cells, effects on the aggregate forming mechanisms (accumulation, replication and infectivity of prion proteins) and on the stability of already formed aggregates. However, it is a unique characteristic of the dendrimers that they have a clear effect on prion plaques in cell-free systems, promoting clearance of pre-existing aggregated prion proteins and thus affecting the aggregates directly.

However, there is still a need to develop effective agents for the treatment of protein aggregate related disease.

DESCRIPTION OF THE INVENTION

The present invention relates to a dendrimer conjugate formed between a dendrimer and a protein solubilizing substance, the conjugate - upon treatment of protein aggregates with the dendrimer conjugate – causing an increase in the protease sensitivity of the protein aggregates over that obtained upon treatment of protein aggregates under the same treatment conditions with a physical mixture of the dendrimer and protein solubilising substance, the physical mixture containing the same molar ratio of the protein solubilizing substance to the dendrimer as that in the dendrimer conjugate, and the increase being evidenced by a protease assay as described herein.

The present invention concerns dendrimer conjugates wherein the protease sensitivity is increased by a factor of more than 1 such as, e.g., at least 1.5 or at least 2.

Description of Schemes

Scheme 1: General structure of two types of commercially available dendrimers, PPI dendrimers (DAB dendrimers) and PAMAM dendrimers, showing the concept of different generations and some examples of useful types of protein solubilising substances which can be coupled to the surface primary amines of the dendrimers.

Scheme 2: Two examples of dendrimer conjugates both containing one free primary amino group on the surface, deriving from the attachment of the dendrimer to the solid phase during synthesis.
Top: Guanidine substituted PAMAM 3rd generation dendrimer conjugate.
Bottom: Sulfonylurea substituted DAB 4th generation dendrimer conjugate.

Definitions and Abbreviations used in the text

A *dendrimer* is a molecule with a structure that extends from one or more core points through multiple generations of successive layers, with each layer having one or more branching points, to end in equivalent surface groups. They can be globular (spherical) or tree-shaped.

A *PPI dendrimer* is defined as a dendrimer consisting of poly(propyleneimine) layers built on a diaminobutane core unit. PPI dendrimers are commercially available and have well-studied physical and chemical properties.

A *PAMAM dendrimer* is defined as a dendrimer consisting of poly(amidoamine) layers built on a ethylenediamine core unit. PAMAM dendrimers are commercially available and have well-studied physical and chemical properties.

The *surface groups* of a dendrimer are those groups which appear at the end of the branches of the dendrimer. They usually occupy the outer surface of the dendrimer structure and as such, they govern the intermolecular interactions of the dendrimer (*e.g.* with solvents). The interaction of a dendrimer with another molecule usually occurs *via* the surface groups.

A *solid phase support* is an insoluble polymer which is functionalised to allow reagents to be bound to its surface *via* a linker entity. Solid phase chemistry simplifies the synthesis and isolation of products, and is commonly used in techniques such as HTS and combinatorial chemistry.

A *linker* is defined as a bifunctional reagent containing an anchor group to the solid phase support and an anchor group to the reagent. The anchor moieties of a linker are joined to the solid phase and substrate.

A *conjugate* is defined as an association of two or more compounds which are covalently bound together to form a new compound. Bonding occurs between the compounds so that the structure of the conjugate can be determined chemically or spectroscopically. A dendrimer conjugate is a conjugate in which one of the compounds is a dendrimer.

A *chaotrope* is a substance which destabilises the structure of a protein in solution. Chaotropes break down the hydrogen-bonded network between water molecules, allowing macromolecules more structural freedom and encouraging protein extension and denaturation.

When used in relation to the current invention, a *protein solubilising substance* is taken to mean a substance which acts upon (insoluble) protein aggregates to make them soluble in a reaction medium. Solubilising such aggregates makes them susceptible to proteases and may give beneficial effects on protein aggregate related diseases.

A *protein denaturant* is a substance which alters the secondary or tertiary structure of a protein, a process which usually destroys or reduces its activity. It is thought that they operate by disrupting non-covalent interactions within the protein.

A *protein aggregate* is an insoluble collection of proteins which have altered properties from their natural state. They often have conformation which differs from their normally soluble state. Such aggregates may be composed of nonbranching fibrillar proteins containing proteins with a β-sheet conformation. Protein aggregates are also known as "plaques".

A *protein aggregate related disease* is a disease which is linked to protein aggregates. The precise relationship between the protein aggregates and the protein aggregate related diseases is yet unclear, but they may be a symptom, a cause or another factor related to the disease. Protein aggregate related diseases are characterised in that they are neurodegenerative diseases, they are all substantially incurable, and are all devastating or fatal.

A *prion-related disease* is a protein aggregate related disease which is characterised by a build-up of insoluble prion protein in an infected animal. Prion-related diseases include new variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy.

An *amyloid-related disease* is a protein aggregate related disease which is characterised by a build-up of insoluble amyloid protein in an infected animal. Alzheimers disease is an example of an amyloid-related disease.

A *physical mixture* of two or more components is a mixture formed by combining the components in solid, liquid or solution phase. No covalent bonds are formed between the components of a physical mixture, and only weak intermolecular forces exist (e.g. H-bonds, van der Waals forces).

The terms "*reduce infectivity*" or "*disinfect*" are used here to mean reducing the ability of an entity to cause a disease. In this invention, the entities are protein aggregates and the diseases are the protein aggregate related diseases.

A *broad spectrum protease* is a protease which acts on a wide range of proteins (*e.g.* a non-specific protease).

Within the current invention, *protease sensitivity* is used to denote the susceptibility of a protein aggregate to a protease enzyme. Normally insoluble protein aggregates will only react with a protease if first solubilised. Hence, the effectiveness of a specific substance can be assessed by treating the protein aggregate with the substance, reacting the mixture with a broad spectrum protease and then performing a blotting assay to determine the amount of protein aggregate which remains.

ApoA1	apoliprotein A1
apoE	apoliprotein E
APP	amyloid precursor protein
BSE	bovine spongiform encephalopathy
ELISA	enzyme linked immunosorbent assay
HTS	High throughput screening
IgGL	immunoglobulin G
PAMAM	poly(amidoamine)
PEGA	polyethylene glycoldimethylacrylamide copolymer
PEI	poly(ethyleneimine)
PPI	poly(propyleneimine)
PrP	prion protein
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase

Detailed description

As mentioned above, the present invention relates to a dendrimer conjugate formed between a dendrimer and a protein solubilizing substance, the conjugate - upon treatment of protein aggregates with the dendrimer conjugate – causing an increase in the protease sensitivity of the protein aggregates over that obtained upon treatment of protein aggregates under the same treatment conditions with a physical mixture of the dendrimer and protein solubilising substance, the physical mixture containing the same molar ratio of the protein solubilizing substance to the dendrimer as that in the dendrimer conjugate, and the increase being evidenced by a protease assay as described herein.

The present invention concerns dendrimer conjugates wherein the protease sensitivity is increased by a factor of more than 1 such as, e.g., at least 1.5 or at least 2.

The present invention discloses a new type of dendrimer conjugate (also denoted decorated dendrimers) that are characterised by having a high density of very efficient protein solubilising groups on their surface. The protein solubilising groups are formed by covalently binding of the dendrimer with one or more same or different protein solubilising substances.

Dendrimers of a number of well-known and well-described types at a generation number that allows substantial definition of the dendrimer are used as scaffolds upon which various protein solubilising substances are attached by facile, one- or few-step syntheses, said protein solubilising substances being selected on the basis of their ability to solubilise protein agggregates. Therefore, dendrimer conjugates according to the invention have the structure:

$D(R)_n$

wherein
D is the dendrimer
R is a radical of the protein solubilising substance which may be the same or different, and
n is an integer greater than 1.

Protein solubilising substance

As mentioned above, a dendrimer conjugate according to the invention contains a protein solubilising substance. Typically, the protein solubilizing substance is a protein denaturant which is selected from the group consisting of ureas, thioureas, sulfonylureas, semicarbazides, hydrazides, thiosemicarbazides, guanidines and chaotropes.

An interesting class of such protein solubilizing substance to be present on the dendrimer surface is derived from chaotropes and from variants thereof, including, but not limited to cationic chaotropes.

Such substances include primary amines, guanidinium groups, thiocyanates and urea groups as well as other known chaotropic groups.

Specific protein solubilizing substances of the dendrimer conjugates of the present invention include amines, guanidinium groups, thiocyanates and urea groups as well as

other known chaotropic groups as *e.g.* thiourea, sulfonylurea, hydrazide, semicarbazide and thiosemicarbazide. In one version of the invention these groups are combined as illustrated in scheme 1 to provide a certain combination and a certain spatial organisation of H-bond forming and hydrophobic and hydrophilic protein solubilizing substances. This is achieved by the combination of terminal, cationic protein solubilizing substances, including guanidines and amines with other functional groups linking the whole protein solubilizing substance to the dendrimer surface.

A suitable protein solubilizing substance may be the repeating unit of a dendrimer itself. However, normally the protein solubilizing substance is different from the repeating unit of the dendrimer to which the protein solubilizing substance is covalently bound. Otherwise the conjugate obtained is merely the next generation of dendrimer.

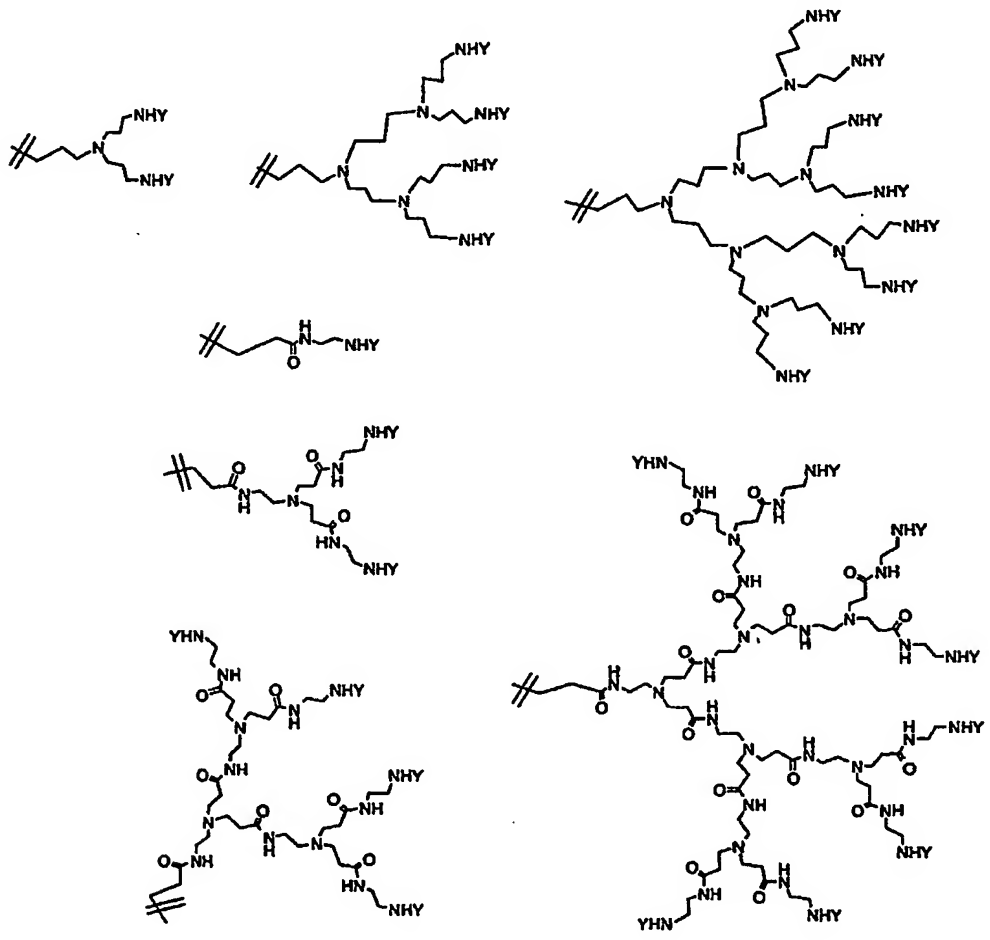
In specific embodiments of the invention the structure of the dendrimer conjugates are illustrated in scheme 1. They provide dendrimer conjugates with a suitable combination and a spatial organisation of H-bond forming and hydrophobic and hydrophilic protein solubilising substances, achieved by the combination of terminal, cationic protein solubilizing substances, including guanidines and amines ("Z" in scheme 1) with another protein solubilizing substance ("Y" in scheme 1). In addition to furnishing the dendrimer surface with cationic protein solubilising substances adjacent to other protein solubilising substances, it is also clear that the listed "Y" groups lead to a different surface distribution of cationic protein solubilising substances than the distribution seen on a conventional cationic dendrimer.

Particular examples of protein solubilizing substances are depicted in scheme 1. As can be seen these substances give the possibility of producing a wide range of different dendrimer conjugates with different densities of terminal protein solubilizing substances in different arrangements and different patterns of hydrogen bonding and hydrophobic regions in the region of the substance ("Y" in scheme 1). This makes possible the synthesis of dendrimer conjugates with finely tuned solubility and protein solubilising charactersitics. The properties of the dendrimer conjugates are also influenced by the dendrimer type used, as PAMAM dendrimers will have a lower charge density in the interior in contrast to PPI dendrimers (polyamines) that will be more highly charged at neutral and acidic pH values.

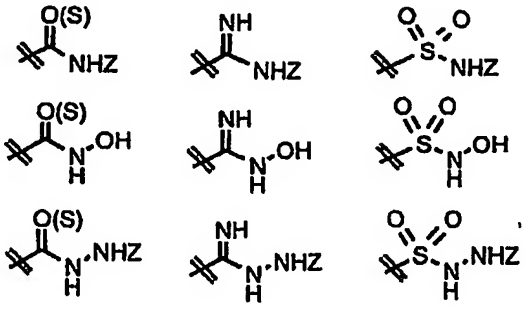
The inclusion of such protein solubilising substances in large numbers on the dendrimers makes such dendrimer conjugates very powerful protein aggregate solubilising agents

that are able to dissolve (partly or completely) protein aggregates at non-cytotoxic concentrations in a matter of few hours.

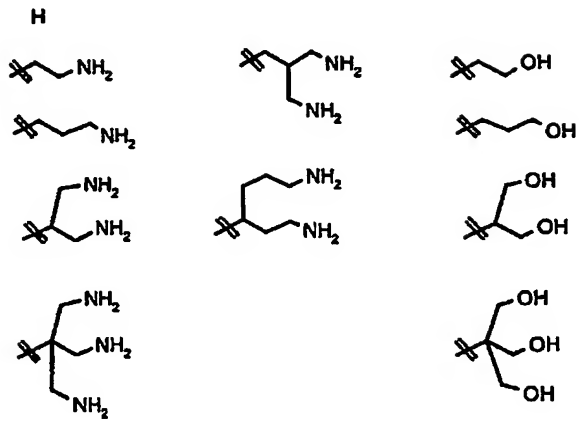
Hence, the present invention relates to dendrimer conjugate as described above, wherein R is



wherein Y is selected from the group consisting of



wherein Z is selected from the group consisting of



Protein solubilizing substances relevant to the current invention are combinations of protein solubilizing substances as depicted in scheme 1 (by N- or O-substitution as appropriate), in which Z belongs to the group of ethylene amine, tris-ethylene amine and the like or hydroxyethylene, glycol or glycerol and the like.

Furthermore dendrimer conjugate according the invention may contain one or more surface groups which are not occupied by a protein solubilising substance. Such surface groups are often amino groups so that, in one embodiment of the current invention, the dendrimer conjugate will contain one unmodified primary amino group on its surface (see scheme 2). This amino group may be a result of the solid phase synthesis where it is bound to the linker group which is used to connect the dendrimer conjugate to the solid phase support. When the dendrimer conjugate is released from the solid phase support, the primary amino group remains.

Dendrimer

The present invention relates to dendrimer conjugates wherein the dendrimer is a multivalent functional dendrimer having a dendritic structure that extends from one or more core points through multiple generations of successive layers, with each layer having one or more branching points, to end in surface groups. The dendrimer conjugate can be represented structurally, and thus the present invention describes dendrimer conjugates, wherein the dendrimer (D) is represented by the formula:

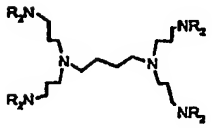
$X-(V)_a-(W)_b$

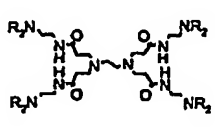
wherein X is a multifunctional segment having one or more branching points,
V is a linker or spacer group, which may be branched or linear
W is a surface group and
a and b are integers such that each linker group V terminates in one or more surface groups W.

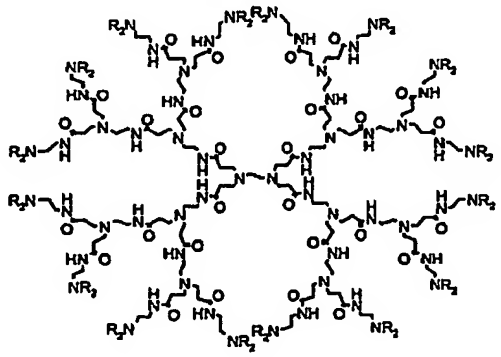
The dendrimer conjugates of the invention can be based on different dendrimer types, which are often commercially available types like PAMAM- denrimers and PPI- (polypropyleneimine) dendrimers, and normally in the range of 2nd - 4th generation, for example 3rd generation.

The dendrimers of the dendrimer conjugates according to the present invention may be globular or tree-shaped. In one embodiment, the generation of the dendrimer ranges from 0 to 20 such as *e.g.* from 1 to 10 or from 2 to 6. In another embodiment, the molecular mass of the unmodified dendrimers according to the present invention lies from 50 to 30000 such as *e.g.* from 100 to 20000 or from 300 to 15000. Furthermore, the dendrimer conjugates of the present invention contain dendrimers in which the number of surface groups on the dendrimer lies between 2 and 256 such as *e.g.* between 2 and 64, between 4 and 32 or between 8 and 32, such as *e.g.* 4, 8, 16, 32 or 64. Dendrimer conjugates according to the present invention are such that wherein the surface groups of the dendrimer are amine functionalities. The current invention also relates to dendrimer conjugates, wherein the dendrimer is a PPI dendrimer or a PEI dendrimer or a PAMAM dendrimer.

Additionally, the present invention describes dendrimer conjugates wherein the dendrimer has one of the following core structures







wherein R has the same meaning as previously described.

In one embodiment according to the invention, the dendrimer is a conjugate of two or more multivalent functional dendrimers as defined herein.

Use of dendrimer conjugates

A particular use of the dendrimer conjugates of the invention is for solubilisation of protein aggregates of different protein malfolding mediated diseases, including the prion diseases. In other words, the dendrimer conjugates according to the invention are to be used in the treatment of protein aggregate related diseases. Specific protein aggregate related diseases relevant to the invention are selected from the group consisting of Alzheimer's disease, Creutzfeldt-Jakob disease, variant Creutzfeldt-Jakobs disease, fatal familial insomnia, Gerstmann-Sträussler-Sheinker syndrome, PrP-cerebral amyloid angiopathy, scrapie, bovine spongiform encephalopathy, chronic wasting disease, transmissible mink encephalopathy, Pick's disease, Parkinson's disease, amyotrophic lateral sclerosis, frontotemporal dementia, diabetes type II, multiple myeloma-plasma cell dyscrasias, familial amyloidotic polyneuropathy, medullary carcinoma of thyroid, chronic renal failure, congestive heart failure, senile cardiac and systemic amyloidosis, chronic inflammation, atherosclerosis, familial amyloidosis and Huntington's disease.

Furthermore, dendrimer conjugates according to the invention are to be used to used in the treatment of protein aggregate related diseases, wherein the protein of the protein aggregate is selected from the group consisting of APP, Aβ peptide, α1-antichymotrypsin, tau, non-Aβ-component, presenillin 1, presenillin 2, apoE, prion protein including protease resistant prion protein, SOD, Pick body, α-synuclein, anylin, IgGL-chain, transthyretin, procalcitonin, β2-microglobulin, atrial natriuretic factor, serum amyloid A, ApoA1, Gelsolin and Huntingtin. In a particular embodiment, the protein aggregate related disease is a prion-related disease. In a further embodiment, the protein aggregate related disease is an amyloid-related disease.

The current invention also discloses a method for treating, preventing and/or diagnosing a protein aggregate related disease in a subject, the method comprising administering to the subject in need thereof a sufficient amount of a dendrimer conjugate. Also disclosed is the use of dendrimer conjugates according to the invention in the preparation of a medicament for use in the treatment, prophylaxis and/or diagnosis of protein aggregate related diseases.

Another use of the dendrimer conjugates of the invention is for treatment of cells or animals, including humans to prevent the build-up of protein aggregates and to promote the clearance of already formed protein aggregates. Therefore, a method of preventing the formation of protein aggregates in cells or animals is disclosed, the method comprising the treatment of cells or animals with a dendrimer conjugate according to the present invention. A particular use of dendrimer conjugates of the present invention is in the treatment or prophylaxis of prion diseases. A further use of dendrimer conjugates according to the invention is to reduce the infectivity of prion proteins. A typical example of this is the solubilisation of scrapie prion protein aggregates in a homogenate from a sheep brain sample from a scrapie-affected sheep.

It is envisaged that different strains of prions (or other kinds of protein aggregates) will have different susceptibilities towards the solubilising effect of the dendrimer conjugate. In particular, sheep prions derived from bovine spongiform encephalopathy plaques (upon exposure of sheep to the BSE agent) will be discernible from scrapie prion protein aggregates in their susceptibility to solubilisation by dendrimer conjugate. To detect these differences in susceptibility to solubilisation a method is disclosed which comprises incubating the dendrimer conjugate-treated prion protein aggregates with a broad spectrum protease, as *e.g.* proteinase K for a suitable time and then detecting remaining prion protein by SDS-PAGE and immunoblotting with prion-specific antibodies e.g. by the Prionics assay as known to a person skilled in the art. Susceptible prion strains will then disappear faster than less susceptible prion strains.

Hence, the dendrimer conjugates according to the invention are to be used for identifying prion protein aggregates. Furthermore, the dendrimer conjugates of the invention are to be used for classifying the protein aggregates into specific strains according to their susceptibility to the treatment described below. A method of identifying and/or classifying protein aggregates in a mammal is also disclosed, the method comprising the steps of:

a) treating the protein aggregates with a dendrimer conjugate

b) analysing one or more products of step a)

In one embodiment of the above method according to the invention, step b) comprises the steps of:

I. incubating the treated protein aggregates from step a) with a broad spectrum protease such as *e.g.* proteinase K

II. detecting remaining protein aggregates by one or more methods selected from the group comprising: SDS-PAGE and immunoblotting with protein-specific antibodies, ELISA, immunoelectrophoresis and immunohistochemistry.

In another embodiment the method comprises using conformationally sensitive antibodies that will only react with unfolded protein; in this type of assay susceptible strains will give rise to a signal while less susceptible strains will give rise to less signal. In other words, in a second embodiment of the above method, step b) of the method comprises incubating the treated protein aggregates from step a) with an antibody which is sensitive to changes in the structure of a protein present in the protein aggregate. In a further embodiment, the dendrimer conjugate itself is labelled, for example with a suitable conformationally sensitive fluorophore enabling the sensitive detection of changes in dendrimer spatial structure, which is believed to occur in a strain specific way upon contacting the dendrimer with prion protein agregates of different strains or types.

Additionally, the method of identifying and/or classifying protein aggregates may additionally comprise the step of further treating the treated protein aggregates from step a) with a protein denaturant such as *e.g.* urea between steps a) and b). This serves to further solubilise an aggregate before the protease treatment and such an additional step discriminates more effectively between protein aggregates from two different sources.

The method of identifying and/or classifying protein aggregates according to the invention may further comprise the steps of

i) repeating steps a) and b) with a different dendrimer conjugate, and

ii) optionally comparing results from the dendrimer conjugates to obtain information on the origin of the protein aggregates.

Normally step ii) is comprised in the method.

An alternative use of the dendrimer conjugates of the present invention is for the decontamination of surfaces, medicines, food, devices, tools and feed-stuff by treatment with dendrimer conjugates to remove or reduce substantially prion infectivity. Hence, a method is disclosed for disinfecting an object, the method comprising contacting the object with a composition containing a dendrimer conjugate according to the invention.

Additionally, a method for removing protein aggregates from food that originates from an animal is related, the method comprising contacting the food with a composition containing a dendrimer conjugate. The dendrimer conjugates of the invention may be used in the disinfection of material which has been contaminated with protein aggregates.

Synthesis of Dendrimer Conjugates

Methods for the synthesis of dendrimer conjugates are also included in this invention. As a general method for synthesising dendrimer conjugates, the dendrimer is first coupled to a suitable solid phase including polystyrene-based resins or PEGA resins through a suitable, selectively cleavable linker moiety. In other words, the invention also relates to a method for the preparation of a dendrimer conjugate, wherein the preparation is carried out while the dendrimer is grafted to a solid phase support through a linker entity. In a particular embodiment of the preparation method according to the current invention, the linker entity is an acid labile linker, such as *e.g.* chlorotritylchloride, Wang, Rink, Sieber or related linkers. In a further embodiment, solid phase support is selected from the group comprising polystyrene, modified polystyrene and PEGA. Once bound to the solid phase , the dendrimer is then treated with a precursor of the desired protein solubilising group. The final dendrimer conjugate is cleaved off the solid phase, and eventual protective groups are cleaved off either during cleavage from solid phase or subsequently. This method of synthesis is amenable to combinatorial chemistry and library screening (HTS) for activity against protein aggregates.

The dendrimer conjugates of the present invention are substantially soluble in water or aqueous buffers and interesting dendrimer conjugates carry a positive net charge at neutral pH. Furthermore, by presenting a high number of protein solubilising groups (chaotropes or other types) on their surfaces, said dendrimers achieve a substantially increased protein aggregate solubilising effect compared to the same solubilising substance employed as a solibliser on its own. It is furthermore possible by specific combinations of the Y and Z groups of the surface groups to fine-tune the dendrimer conjugates of the invention to exhibit a desired degree and specificity of protein aggregate solubilising ability.

EXAMPLES

The following examples provide evidence of the feasibility of the invention but are not meant to limit the invention to the uses and the embodiments presented in the examples.

General: Synthesis of isothiocyanates.
Carbondisulfide (10 equiv) is dissolved in DCM and 1.1 equiv peptide coupling reagent (e.g. PyBOP or TFFH) is added followed by 1 equiv protected amine and 3 equiv NMM. The mixture is stirred for 30 min at r.t. DCM and carbondisulfide is evaporated in vacuo and the crude isothiocyanate is ready for further synthesis.

EXAMPLE 1: Solid phase synthesis of thiourea-dendrimer conjugates.
Amino terminated dendrimer (1.5 equiv) is added to a chlorotrityl-chloride resin (1 equiv) in DCM. The suspension is shaken for 2h at r.t. Residual chlorotrityl groups are capped with a DCM/methanol/NMM 17:2:1 mixture. The resin is washed with DCM (5 times) and NMP (5 times). TNBSA test shows positive. The amino terminated dendrimer bound to the chlorotrityl-chloride resin is suspended in NMP and an adequately protected isothiocyanate (5 equiv relative to numbers of surface amines on the dendrimer) and the mixture is shaken for 2 days at r.t. The resin is washed with NMP (10 times) and DCM (5 times). TNBSA test shows negative. The dendrimer conjugate is deprotected and cleaved off the resin with 50 % TFA in DCM for 2 h at r.t. The dendrimer conjugate is triturated with diethyl ether.

EXAMPLE 2: Solid phase synthesis of guanidine-dendrimer conjugates.
The amino-terminated dendrimer bound to a chlorotrityl-chloride resin is prepared as in Example 1 and suspended in NMP and N-Boc-protected S-methyl-isothiourea (5 equiv relative to number of surface amines on the dendrimer) is added. The suspension is shaken for 16h at 50°C. The resin is washed with NMP (10 times) and DCM (5 times). TNBSA test shows negative. The dendrimer conjugate is deprotected and cleaved off the resin with 50% TFA/DCM. The dendrimer conjugate is triturated with diethyl ether.

EXAMPLE 3: Solid phase synthesis of sulfonylurea-dendrimer conjugates.
Amino terminated dendrimer bound to a chlorotrityl-chloride resin is prepared as in Example 1 and resuspended in dry DCM/pyridine 1:1 mixture and sulfurylchloride (5 equivalents relative to number of surface amines on the dendrimer). The mixture is shaken for 3h at r.t. The resin is washed with dry DCM (3 times), and a suitably protected amine (5 equiv relative to number of surface amines) is added and the suspension is

shaken overnight at r.t. The dendrimer conjugate is cleaved off the resin together with protecting groups at the dendrimer with 50% TFA in DCM for 2h at r.t. The dendrimer conjugate is triturated with diethyl ether.

EXAMPLE 4: Increase of protease susceptibility of hamster prion protein aggregates after treatment with dendrimer conjugates.

Hamsters are inoculated intracereberally with 263K hamster-adapted scrapie strain and monitored closely until onset of clear clinical signs. Then the animals are killed and the brain is retrieved and homogenised .The brain homogenate is subjected to proteinase K-treatment followed by immunoblotting developed by Prionics 6H4 monoclonal antibody and peroxidase-conjugated anti mouse secondary antibodies visualised by a chemiluminescent substrate.

To test the applicability of dendrimer conjugates for sensitising prion aggregates towards proteinase K, the brain homogenate is first incubated for various length of time with the dendrimer conjugate in question in a range of concentrations including 10 microg/ml. Incubation is performed at different pH values, including acidic pH values and in the presence or absence of mild detergents like NP40 and at room temperature or at 37 °C.
Control incubations include:
-no addition of dendrimer or dendrimer conjugate or chaotrope ,
-addition of the unconjugated dendrimer,
-addition of the chaotrope corresponding to the protein solubilising substance on the dendrimer
-addition of said chaotrope plus unconjugated dendrimer

Following this incubation, samples are neutralised if necessary and Sarkosyl is added, for example at a concentration of 4%.
Hereafter the sample is subjected to proteinase K (total protein/enzyme ratio typically 25/1) for 1 hour at 37°C, stopping the protease treatment by PMSF in ethanol. Then samples are immunoblotted as above. Some samples are subjected to treatment with traditional denaturants as e.g. urea before treatment with proteinase K.
Results show that brain homogenates treated by dendrimer conjugates are dramatically more susceptible to proteinase K-degradation than non-treated homogenates as seen by the disappearance of 6H4-reactive bands after proteinase K-treatment and immunoblotting.

EXAMPLE 5: Decreased infectivity of dendrimer conjugate-treated aggregated prion protein compared to untreated prion protein aggregates.

Brain homogenates obtained as in the previous example are inoculated intracereberally into hamsters and the development of clinical disease is followed. It will be seen that brain homogenates first treated by dendrimer conjugates are considerably less efficient in transferring disease than non-treated dendrimers,

EXAMPLE 6: Curing of PrP^{Sc} - "infected" cells of protease resistan prion protein aggregates by treatment of cell cultures with dendrimer conjugates.

The persistenly scrapie-infected mouse neuroblastoma-derived cell line ScN2a are used. Cells are maintained in culture and exposed to various types of dendrimer conjugates at different concentrations and for different time periods to assess the cytotoxicity of the compounds. Cytotoxicity is assessed by measurement of formazan dye reduction (MTS) and by observation of cell morphology. It is expected that dendrimer conjugates at non-cytotoxic concentrations and after relatively short exposure times, as for example 5 hours or even 2 hours will remove protease-resistant prion protein aggregates from the culture as seen by the disappearance of protease-resistant anti-prion protein reactive material after SDS-PAGE and immunoblotting as above.

EXAMPLE 7: Halting a scrapie prion infection in hamsters by treating PrP^{Sc} inoculated hamsters with dendrimer conjugates at various time points after inoculation

Hamsters are inoculated intracerebrally with 263 K as above. At different times after inoculation as well as just before inculation, different groups of animals are injnected intracerebrally with different amounts of different types of dendrimer conjugates. It is expected that an optimal dendrimer conjugate treatment protocol can be identified in which the development of protease resistant prion protein in inculated hamsters is inihibited totally or substantially.

EXAMPLE 8: Differentiating between two types of prion protein aggregates by their different susceptibility towards dendrimer conjugates.

Hamsters are inoculated intracerebrally with 263 K and brain homogenates are prepared as above. BSE prion protein aggregates are obtained from brain samples from naturally BSE infected cattle (available at the Danish Veterinary Institute).

Both types of samples are subjected to a range of treatment protocols by dendrimer conjugates and subjected to proteinase treatment and SDS-PAGE immunoblotting as above. Some treatment protocols combine treatment with dendrimer conjugate with a

subsequent solubilising step for example with urea at at high concentration, for example 6 M or 8 M, before the proteinase treatment.

It is expected that an optimal group of treatment protocols will lead to immunoblots showing substantial differences in the proteinase susceptibility of 263K as compared to BSE prions, as seen by a clear difference in the intensity of bands on the immunoblot between the two prion types.

CLAIMS

1. A dendrimer conjugate formed between a dendrimer and a protein solubilizing substance, the conjugate - upon treatment of protein aggregates with the dendrimer conjugate – causing an increase in the protease sensitivity of the protein aggregates over that obtained upon treatment of protein aggregates under the same treatment conditions with a physical mixture of the dendrimer and protein solubilising substance, the physical mixture containing the same molar ratio of the protein solubilizing substance to the dendrimer as that in the dendrimer conjugate, and the increase being evidenced by a protease assay as described herein.

2. A dendrimer conjugate according to claim 1, wherein the dendrimer is covalently bound to one or more same or different protein solubilising substances.

3. A dendrimer conjugate according to claim 1 or 2 having the structure

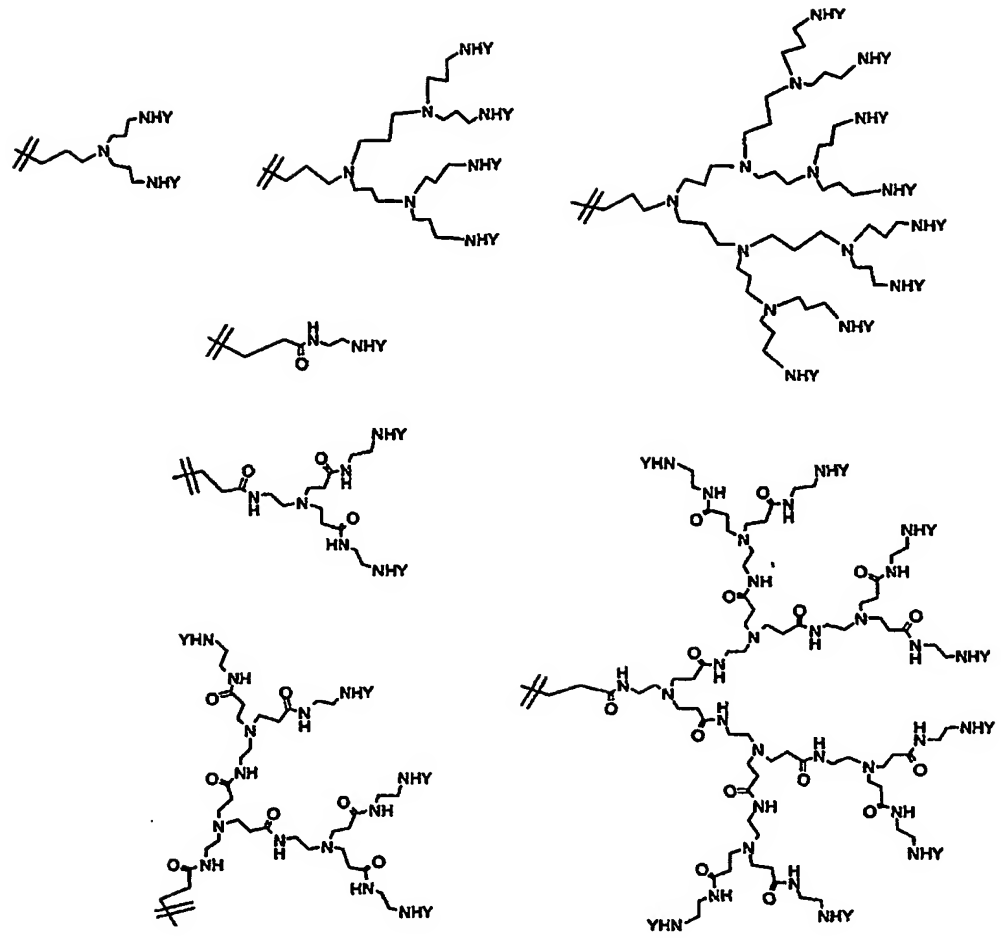
$$D(R)_n$$

wherein
D is the dendrimer
R is a radical of the protein solubilising substance which may be the same or different, and
n is an integer greater than 1.

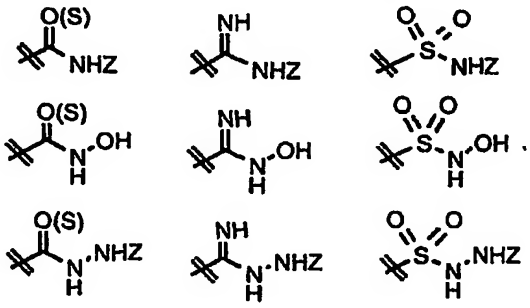
4. A dendrimer conjugate according to any of claims 1-3, wherein the protein solubilising substance is a protein denaturant.

5. A dendrimer conjugate according to claim 4, wherein the protein denaturant is selected from the group consisting of ureas, thioureas, sulfonylureas, semicarbazides, hydrazides, thiosemicarbazides, guanidines and chaotropes.

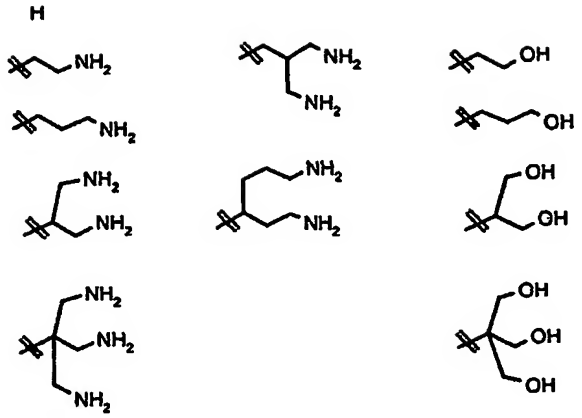
6. A dendrimer conjugate according to claim 3, wherein R is



wherein Y is selected from the group consisting of



wherein Z is selected from the group consisting of



7. A dendrimer conjugate according to any of claims 1-6, wherein the protease sensitivity is increased by a factor of more than 1 such as, e.g., at least 1.5 or at least 2.

8. A dendrimer conjugate according to any of claims 1-7 containing one or more surface groups which are not occupied by a protein solubilising substance

9. A dendrimer conjugate according to any of claims 1-8, wherein the dendrimer (D) is a multivalent functional dendrimer having a dendritic structure that extends from one or more core points through multiple generations of successive layers, with each layer having one or more branching points, to end in surface groups.

10. A dendrimer conjugate according to claim 9, wherein the dendrimer (D) is represented by the formula:

$$X\text{-}(V)_a\text{-}(W)_b$$

wherein X is a multifunctional segment having one or more branching points,
V is a linker or spacer group, which may be branched or linear
W is a surface group and
a and b are integers such that each linker group V terminates in one or more surface groups W.

11. A dendrimer conjugate according to any of claims 9 or 10, wherein the dendrimer is globular or tree-shaped.

12. A dendrimer conjugate according to any of claims 9-11, wherein the generation of the

dendrimer ranges from 0 to 20 such as *e.g.* from 1 to 10 or from 2 to 6.

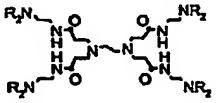
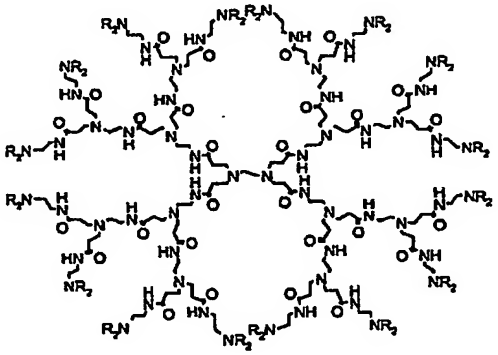
13. A dendrimer conjugate according to any of claims 9-12, wherein the molecular mass of the unmodified dendrimers lies from 50 to 30000 such as *e.g.* from 100 to 20000 or from 300 to 15000.

14. A dendrimer conjugate according to any of claims 9-13, wherein the number of surface groups on the dendrimer lies between 2 and 256 such as *e.g.* between 2 and 64, between 4 and 32 or between 8 and 32, such as *e.g.* 4, 8, 16, 32 or 64.

15. A dendrimer conjugate according to any of claims 9-14, wherein the surface groups of the dendrimer (D) are amine functionalities.

16. A dendrimer conjugate according to any of claims 9-15, wherein the dendrimer is a PPI dendrimer or a PEI dendrimer or a PAMAM dendrimer.

17. A dendrimer conjugate according to any of claims 9-16, wherein the dendrimer has one of the following core structures

wherein R has the same meaning as in claim 3.

18. A dendrimer conjugate according to any of claims 1-8, wherein the dendrimer (D) is a conjugate of two or more multivalent functional dendrimers as defined in claims 9-17.

19. Use of a dendrimer conjugate according to any of claims 1-18 in the treatment of protein aggregate related diseases.

20. Use according to claim 19, wherein the protein aggregate related disease is selected

from the group consisting of Alzheimer's disease, Creutzfeldt-Jakob disease, variant Creutzfeldt-Jakobs disease, fatal familial insomnia, Gerstmann-Sträussler-Sheinker syndrome, PrP-cerebral amyloid angiopathy, scrapie, bovine spongiform encephalopathy, chronic wasting disease, transmissible mink encephalopathy, Pick's disease, Parkinson's disease, amyotrophic lateral sclerosis, frontotemporal dementia, diabetes type II, multiple myeloma-plasma cell dyscrasias, familial amyloidotic polyneuropathy, medullary carcinoma of thyroid, chronic renal failure, congestive heart failure, senile cardiac and systemic amyloidosis, chronic inflammation, atherosclerosis, familial amyloidosis and Huntington's disease.

21. Use according to any of claims 19 or 20, wherein the protein of the protein aggregate is selected from the group consisting of APP, Aβ peptide, α1-antichymotrypsin, tau, non-Aβ-component, presenillin 1, presenillin 2, apoE, prion protein including protease resistant prion protein, SOD, Pick body, α-synuclein, anylin, IgGL-chain, transthyretin, procalcitonin, β2-microglobulin, atrial natriuretic factor, serum amyloid A, ApoA1, Gelsolin and Huntingtin

22. Use of dendrimer conjugates according to any of claims 19 or 20, wherein the protein aggregate related disease is a prion-related disease

23. Use of dendrimer conjugates according to any of claims 19 or 20, wherein the protein aggregate related disease is an amyloid-related disease.

24. Use of dendrimer conjugates according to any of claims 1-18 to reduce the infectivity of prion proteins.

25. Use of dendrimer conjugates according to any of claims 1-18 in disinfection of material which has been contaminated with protein aggregates.

26. A method of identifying and/or classifying protein aggregates in a mammal, the method comprising the steps of:

a) treating the protein aggregates with a dendrimer conjugate as defined in claims 1-18

b) analysing one or more products of step a)

27. The method according to claim 26 wherein step b) comprises the steps of

I. incubating the treated protein aggregates from step a) with a broad spectrum protease such as *e.g.* proteinase K

II. detecting remaining protein aggregates by one or more methods selected from the group comprising: SDS-PAGE and immunoblotting with protein-specific antibodies, ELISA, immunoelectrophoresis and immunohistochemistry.

28. The method according to claim 26 wherein step b) comprises incubating the treated protein aggregates from step a) with an antibody which is sensitive to changes in the structure of a protein present in the protein aggregate.

29. The method according to claim 26 additionally comprising the step of further treating the treated protein aggregates from step a) with a protein denaturant such as *e.g.* urea between steps a) and b).

30. The method according to claim 26 further comprising the steps of

i) repeating steps a) and b) with a different dendrimer conjugate, and
ii) optionally comparing results from the dendrimer conjugates to obtain information on the origin of the protein aggregates.

31. Use of dendrimer conjugates according to any of claims 1-18 for identifying prion protein aggregates.

32. Use of dendrimer conjugates according to any of claims 1-18 for classifying the protein aggregates into specific strains according to their susceptibility to the treatment described in claim 26.

33. A method of preventing the formation of protein aggregates in cells or animals, the method comprising the treatment of cells or animals with a dendrimer conjugate according to any of claims 1-18.

34. A method for disinfecting an object, the method comprising contacting the object with a composition containing a dendrimer conjugate according to any of claims 1-18.

35. A method for removing protein aggregates from food that originates from an animal, the method comprising contacting the food with a composition containing a dendrimer conjugate according to any of claims 1-18.

36. A method for treating, preventing and/or diagnosing a protein aggregate related disease in a subject, the method comprising administering to the subject in need thereof a sufficient amount of a dendrimer conjugate according to any of claims 1-18.

37. Use of dendrimer conjugates according to any of claims 1-18 in the preparation of a medicament for use in the treatment, prophylaxis and/or diagnosis of protein aggregate related diseases.

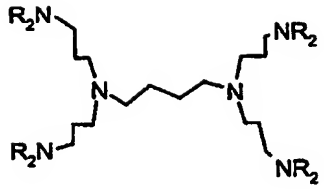
38. A method for the preparation of a dendrimer conjugate according to any of claims 1-18 wherein the preparation is carried out while the dendrimer (D) is grafted to a solid phase support through a linker entity.

39. A method according to claim 38 wherein the linker entity is an acid labile linker, such as *e.g.* chlorotritylchloride, Wang, Rink, Sieber or related linkers.

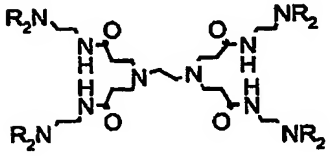
40. A method according to claim 38 wherein the solid phase support is selected from the group comprising polystyrene, modified polystyrene and PEGA.

Scheme 1:

PPI dendrimers



PAMAM dendrimers



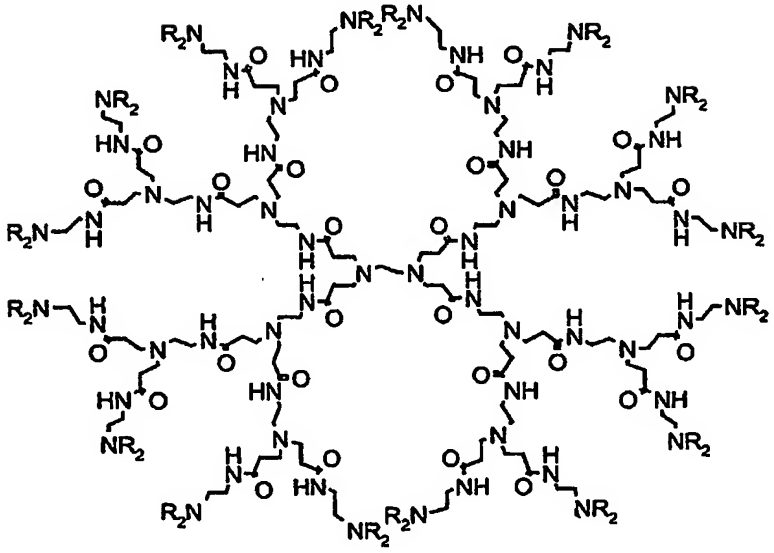
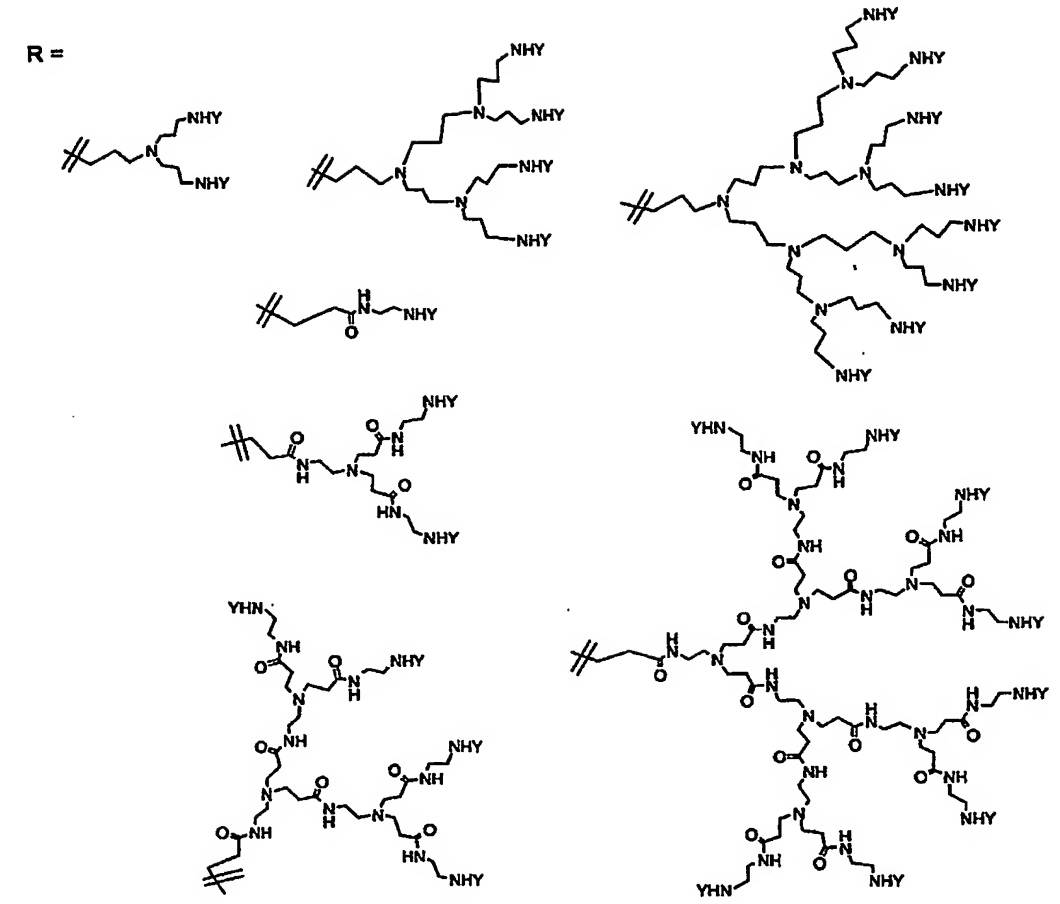


FIGURE 1

Scheme 1 continued



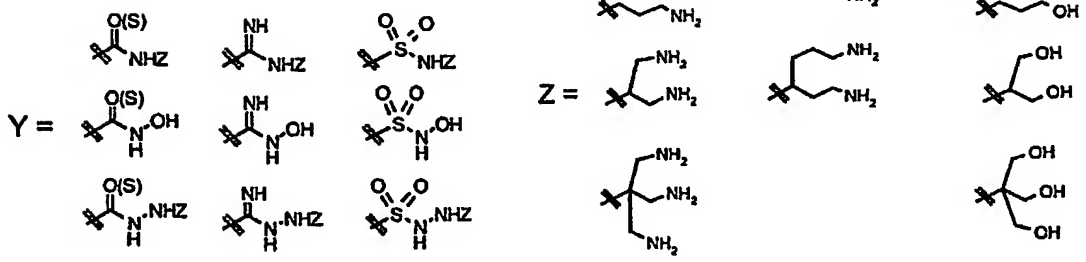


FIGURE 1A.

Scheme 2:

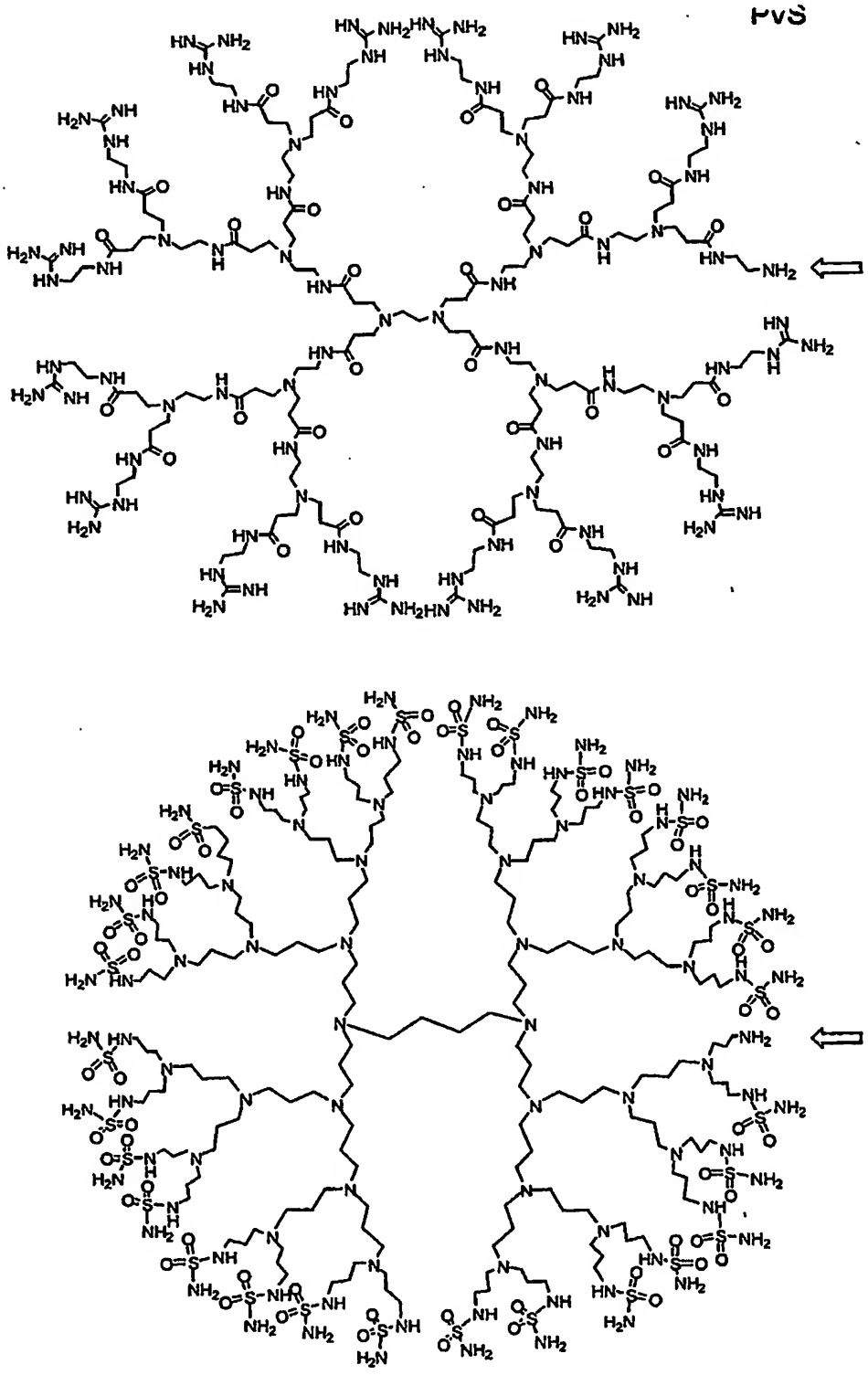


FIGURE 2.